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RATTLESNAKE NEUROTOXIN STRUCTURE, MECHANISM OF ACTION, IMMUNOLOGY AND MOLECULAR BIOLOGY

FINAL REPORT

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SEPTEMBER 10, 1992

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-89-C-9007

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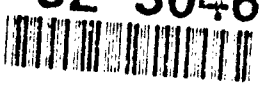


SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Wyoming 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Box 3944, University Station Laramie, Wyoming 82071-3944	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-89-C-9007
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21702-5012	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 62787A PROJECT NO: 3M1-62787A871 TASK NO: AA WORK UNIT ACCESSION NO: DA317821

11. TITLE (Include Security Classification)
(u) Rattlesnake neurotoxin structure, mechanism of action, immunology and molecular biology

12. PERSONAL AUTHOR(S)
KAISER, Ivan I.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 1 Mar 89 TO 31 Aug 92	1992 SEPT 10	60

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Crotoxin, presynaptic neurotoxins, rattlesnake toxins, natural toxins, RA 1
06	03		
06	07		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

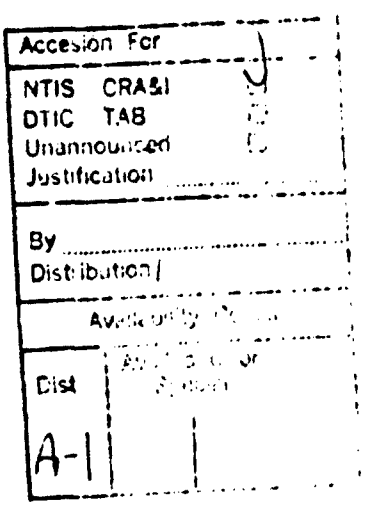
This project was designed to provide a better understanding of rattlesnake neurotoxin structure, mechanism of action, immunology and molecular biology. We demonstrated the structural and functional similarities of all rattlesnake neurotoxins, showed that the acidic subunit plays more than a chaperone role for the basic subunit and is clearly involved in the targeting of crotoxin. Sequencing studies have provided the primary sequence of the basic subunit of Mojave toxin, identified phospholipase A_2-like molecules in Bothrops asper venom, identified isoforms of notexin, and expanded our phospholipase A_2 sequence database. In studies on the mechanism of action we identified and partially characterized the binding properties of crotoxin receptors on brain synaptosomes. In collaborative experiments we examined crotoxin's neuromuscular blocking properties on isolated phrenic nerve-hemidiaphragm preparations under a variety of conditions and have preliminary evidence suggesting that crotoxin does not become internalized to exert its poisoning effects. Characterization of four different monoclonal antibodies raised against the basic subunit of crotoxin were (over)

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	21 ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Fran Bostian	22b TELEPHONE (Include Area Code) 301-619-7326 22c OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued)

examined for their cross-reactivity and shown to react with unique, continuous epitopes. Polyclonal antibodies raised to the same antigen indicated that four regions of the basic subunit were antigenic. In collaborative experiments we have successfully constructed both cDNA and genomic libraries of Crotalus scutulatus scutulatus. Acidic and basic subunit clones have been identified in both libraries. Both cDNA clones and the acidic subunit genomic clone have been sequenced. The basic subunit cDNA clone has been inserted into an E. coli expression vector and shown to be expressed upon induction.



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JC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Susan J. Tucci 9/4/92

PI - Signature DATE

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INTRODUCTION
NATURE OF THE PROBLEM

This contract was designed to provide a better understanding of rattlesnake neurotoxin structure, mechanism of action, immunology and molecular biology. When written in 1988, evidence had accumulated suggesting great similarities between crotoxin, Mojave toxin from C. s. scutulatus, concolor toxin from C. v. concolor, and vegrandis toxin from C. vegrandis in both structure and function (Aird and Kaiser, 1985; Kaiser et al., 1986; Kaiser and Aird, 1987). Our recent studies and those of others (Lennon and Kaiser, 1990; Hawgood and Bon, 1991; Henderson and Bieber, 1986) have now convincingly demonstrated the structural and functional similarities of all rattlesnake neurotoxins. This group of presynaptic neurotoxins consists of moderately toxic basic phospholipases A_2 and a non-covalently associated non-toxic, acidic subunit. The acidic subunit is required for full toxicity, but had no other identified function until we demonstrated in work supported by this contract that acidic subunit plays more than a chaperone role for the basic subunit and that it, in concert with the basic subunit, facilitates the targeting of crotoxin. Intact toxin is probably bound to the target site before release of the acidic subunit. What promotes dissociation of the subunits is unclear. Studies proposed with the phospholipases A_2 from B. asper and other basic phospholipases, were to determine whether monomeric, basic phospholipases A_2 could associate with the acidic subunit of crotoxin and whether there was any induced "neurotoxin action" in a normally non-neurotoxic molecule. In addition, sequencing of these molecules expanded our phospholipase A_2 data base and made available a venom derived monomeric, lipase for trial crystallization studies. Sequencing of the basic subunit of Mojave toxin was also proposed in this application, to facilitate our x-ray structural determination of this molecule. Finally, we proposed to prepare highly purified acidic and basic subunit of Mojave toxin, as well as F_{ab} fragments of our neutralizing monoclonal antibodies for crystallization trials. All crystallization trials and x-ray diffraction studies were carried out in the laboratory of Dr. Keith Ward (Naval Research Laboratories). These structural studies would

further enhance our understanding of the higher-ordered structure of the rattlesnake neurotoxins, their subunits, and related presynaptic neurotoxins.

Snake presynaptic toxins such as crotoxin, are known to block neuromuscular transmission by inhibiting the release of acetylcholine from the presynaptic nerve terminal, but the detailed molecular mechanisms have not been established (Chang, 1985). A better understanding of this mechanism is ultimately essential if we are to rationally develop different materials that interfere with the neurotoxin's actions. It has been proposed by Bon et al. (1988) that the negatively charged phospholipids are a possible target for crotoxin. The presence of other phospholipases A_2 with little or no neurotoxicity, suggests that there are specific binding sites of high affinity for neurotoxic phospholipases A_2 on neural membranes, and that no such sites exist for non-neurotoxic lipases (Rehm and Betz, 1982, 1984; Tzeng et al., 1986). In our studies on the mechanism of action, we proposed several approaches to probe crotoxin's neurotoxic mechanism. One involved a search and characterization of a receptor on synaptosomal membranes using ^{125}I-labeled crotoxin and a cross-linking photoaffinity probe. A preliminary report by Tzeng et al. (1986) has described such a receptor, but no characterization was done. A similar membrane protein was reported earlier by Rehm and Betz (1983) for β-bungarotoxin. We were interested in searching for such a receptor moiety, and if found determine its makeup, molecular weight, association characteristics, etc. We were also interested to determine whether the receptor was associated with a higher-ordered binding complex as found for β-bungarotoxin in chick brain membranes (Rehm and Betz, 1984). We proposed visualizing the interaction of crotoxin with nerve terminals in a series of in vivo and in vitro experiments using ^{125}I-labeled crotoxin and electron microscope autoradiographic procedures. It was thought that these studies might provide insight on whether crotoxin is internalized and if so what is the mechanism of internalization (see papers by Black and Dolly, 1986a and b). Finally, bioluminescence methodology described by Israel and Lesbats (1987) had been applied to the measurement of acetylcholine released from isolated synaptosomes. We felt that these techniques might permit the continuous detection of acetylcholine release in physiological solutions and greatly enhance

the usefulness of synaptosomes and tissue culture cells as an assay system for examining the effects of different toxins on acetylcholine release.

Our preparation (Kaiser and Middlebrook, 1988a) of a neutralizing monoclonal antibody against the basic subunit of crotoxin provided a tool to attempt to identify a synthetic peptide fragment that could be used as a non-toxic, synthetic vaccine (Walter, 1986) against crotoxin toxicity. Also, the delineation of the full profile of continuous antigenic sites of the basic subunit of crotoxin using polyclonal antisera could be correlated with their conformational locations, based on the three-dimensional studies of the crotoxin homolog, Mojave toxin by Keith Ward's laboratory (Norden et al., 1987). We argued that these, and related immunological studies, would give us a better understanding of crotoxin and presynaptic neurotoxin immunology.

As noted before, little was known about the biosynthesis of crotalid neurotoxins, or any venom proteins found in reptiles. We have been successful in preparing cDNA and genomic libraries of Crotalus s. scutulatus in lambda phage in collaboration with Dr. Leonard Smith. cDNA clones for both subunits of Mojave toxin have been isolated and sequenced in Leonard Smith's laboratory. We isolated and are currently working with genomic clones containing both subunits of Mojave toxin. These studies will permit manipulation and selective alterations of the crotoxin gene. In addition to the oligonucleotide-directed site-specific mutagenesis studies, designed to generate a non-toxic, antigenic crotoxin homolog, there are a number of structure-function questions concerning the toxin that will be amenable to study using these clones.

BACKGROUND

In earlier work we were concerned with sequencing studies of the basic and acidic subunit of crotoxin. We completed the sequence determination of the basic subunit, and published those results (Aird, Kaiser, Lewis, and Kruggel; 1986 and 1987). Two of the three acidic subunit chains, and 24-residues of the carboxyl-terminus of the third chain were sequenced (Aird, Kaiser, Lewis, and Kruggel, 1985). Repeated attempts to sequence the blocked, amino-terminal end of the B-chain by conventional methods were unsuccessful. In

1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. He was recently able to provide us with the amino-terminus sequence of the B-chain as shown below.

pE-E-D-G-E-I-V-C-G-E-D-D-D-P-C-...

A manuscript describing this work has appeared (Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser, 1990). Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution. Others have unsuccessfully attempted to sequence the acidic subunit for the past fifteen years.

Structural studies on crotoxin, its subunits, and related toxins, have been completed. We examined the spectral properties of four presynaptic neurotoxins from the venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus, and C. v. concolor. These and their subunits were examined by circular dichroism, deconvolution Fourier-transform infrared, and fluorescence spectroscopy. This work has been published (Aird, Steadman, Middaugh, and Kaiser, 1989) which suggested that in general the isolated subunits were decreased slightly in α-helix, while they were increased in β-sheet structure, relative to intact toxins. We initially concluded from these results that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Intact crotoxin, however, when exposed to urea, yields spectra that are virtually identical to control intact crotoxin. These findings suggest that the enhanced fluorescence exhibited by the isolated subunits, as well as the secondary structural changes in α-helix and β-sheet, are artifacts resulting from irreversible structural changes that occur during subunit isolation by urea ion-exchange chromatography.

We examined venoms from the Great Basin rattlesnake (C. v. lutosus), Uracoan rattlesnake (C. vegrandis), Western diamondback rattlesnake (C. atrox), Western diamondback-Mojave rattlesnake (C. s. scutulatus) hybrids, and C. d. collilineatus, for crotoxin-like neurotoxins. Publications resulted from studies on the Uracoan (Kaiser and Aird, 1987); the Great Basin rattlesnake (Aird, Seebart, and Kaiser; 1988), the Western diamondback (Aird, Thirkhill, Seebart, and Kaiser; 1989), and C. d. collilineatus (Lennon and Kaiser,

1990). We also examined the myotoxin fraction from C. v. concolor, as a result of earlier indications that there were some sequence homologies between these smaller peptides and the basic subunit of crotoxin, as well as the existence of myotoxin isoforms. A publication describing these results has been published (Ownby, Aird, and Kaiser, 1988). We further demonstrated multiple myotoxin sequences from the venom of a single prairie rattlesnake (C. v. viridis) in the article by Aird, Kruggel, and Kaiser (1991). Dr. Keith Ward has been conducting x-ray crystallography studies on the the crotoxin homolog, Mojave toxin, which we purified and provided to him. A preliminary report has appeared on this work (Norden, Ward, Kaiser, and Aird; 1987), as well as part of a book chapter authored by Ward (Ward and Pattabiraman, 1990).

Modification studies on purified crotoxin were carried out in attempts to identify structural requirements for toxicity. We have approached this problem chemically and immunologically. Four different chemical cross-linkers and one Lys-specific monofunctional reagent have been used in crotoxin modification studies. We finally concentrated on the most promising of these, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide. We determined that cross-linked crotoxin was non-toxic at i.v. injections up to 2-3 μg/g in mice and concluded that loss of toxicity was due to either subunit cross-linking or modification of essential residues (see BODY and reference by Lennon, Plummer, and Kaiser; 1990).

Our immunological studies have involved polyclonal antisera raised to crotoxin, its subunits, and crotoxin homologs as described in the publication by Kaiser, Middlebrook, Crumrine, and Stevenson (1986). Further, in collaboration with Dr. John Middlebrook, we have isolated four monoclonal antibodies raised to the basic subunit of crotoxin. One is a potent neutralizer of crotoxin's lethality and phospholipase A_2 activity as described in the following publications (Kaiser and Middlebrook, 1988a and b). Middlebrook's laboratory has now prepared anti-idiotype monoclonal antibodies against the neutralizing monoclonal and are examining their potential as crotoxin immunogens. We also examined these for possible phospholipase activity, but found none.

We conducted preliminary experiments using guinea pig brain synaptosomes and ^{3}H-choline release measurements to monitor responses to crotalid neurotoxins. Existing assays and procedures

have proved to be more extensive and involved than originally anticipated. New methodology, employing luminometry was examined, but in our hands has not been satisfactory when used with brain synaptosomes.

Dr. Lance Simpson (Jefferson Medical College) has examined crotoxin for its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. He found the toxin produces concentration-dependent paralysis of transmission with a high temperature dependence. Our crotoxin-neutralizing monoclonal antibody at equimolar concentration, abolishes the toxicity if pre-mixed prior to addition to tissues. If antibody is added to tissues after the toxin has become bound, it no longer affords protection. One of our non-neutralizing monoclonal antibodies at equimolar concentrations to crotoxin, does not delay onset of neuromuscular blockage. A publication describing this work has appeared (Trivedi, Kaiser, Tanaka and Simpson, 1989). More recently Simpson completed a series of studies designed to identify the site at which phospholipase A_2 neurotoxins localize to produce their neuromuscular effects. This work is summarized in the BODY of this report.

Work relating to the cloning of the genes for the crotalid presynaptic neurotoxin had several false starts. In collaboration with Dr. Leonard Smith (USAMRIID), we have now successfully constructed both cDNA and genomic libraries of Crotalus s. scutulatus. Acidic and basic subunit clones have been identified in both libraries. Smith has sequenced three acidic and four basic subunit clones from the cDNA library. We have cloned one of the basic subunit cDNAs into an E. coli expression vector and demonstrated be SDS-PAGE and western blotting that the protein is expressed upon induction. Our laboratory has now cloned the genomic DNA for Mojave toxin acidic and basic subunit genes. Sequencing of the acidic subunit is complete and the basic subunit is nearing completion, as described in the BODY of the report.

PURPOSE OF THE PRESENT WORK

This was a multifaceted approach involving a detailed examination of rattlesnake neurotoxins which has provided us with a better understanding of these molecules. Our goals were to know (1)

more about the primary, secondary, and tertiary structure of rattlesnake neurotoxins and non-toxic phospholipase A_2 molecules; (2) whether crotoxin interacts with a membrane binding receptor; (3) whether crotoxin is internalized at the motor neurons and the mechanism of that internalization; (4) whether an in vitro system for monitoring continuous acetylcholine release from brain synaptosomes is feasible; (5) the full profile of continuous antigenic sites on the basic subunit of crotoxin and possibly the epitope recognized by our neutralizing monoclonal antibody; (6) whether the neutralizing monoclonal antibody F_{ab}-antigen complex is crystallizable; and (7) whether non-toxic crotoxin antigen can be generated by site-specific mutagenesis.

We felt that the above studies would provide insights into methods of defense against rattlesnake neurotoxins, a better understanding of their basic chemistry, mechanism of action, and therapies; and a possible in vitro model system for presynaptic neurotoxin assay and screening of potential therapeutic compounds.

METHODS OF APPROACH

a. Structure. 1. Determine the disulfide bond arrangement in the basic subunit of crotoxin using a combination of chemical and mass spectrometer methods. 2. Determine the primary sequence of the basic subunit of Mojave toxin. 3. Determine the primary sequence of the monomeric, basic phospholipase A_2 from Bothrops asper, and examine its ability to associate with the acidic subunit of crotoxin. We will also provide Dr. Keith Ward (Naval Research Laboratories) with sufficient purified material of this basic phospholipase for crystallization experiments. 4. Prepare highly purified acidic and basic subunits of Mojave toxin for crystallization trials by Ward's laboratory.

b. Mechanism of Action. 1. Prepare ^{125}I-labeled crotoxin which retains its neurotoxicity and phospholipase activity. Use this material in examining (i) its binding to synaptosomal membranes, (ii) cross-linking to specific membrane components, (iii) brain synaptic membranes for high affinity crotoxin binding sites, and (iv) crotoxin's interaction with nerve terminals, using electron microscopic autoradiography. 2. Employ new bioluminescence techniques to monitor continuous acetylcholine release from brain

synaptosomes in vitro, in the absence and presence of purified presynaptic neurotoxins.

c. Immunology. 1. Examine the full profile of continuous antigenic sites on the basic subunit of crotoxin and attempt to identify the epitope recognized by our neutralizing monoclonal antibody. 2. Prepare highly purified F_{ab} fragments of our neutralizing monoclonal antibody for crystallization attempts of the F_{ab}-antigen complex by Keith Ward.

d. Continue our work on the **molecular biology** of crotoxin, directed to the eventual production of a non-toxic crotoxin antigen by site-specific mutagenesis.

BODY OF REPORT

METHODS, RESULTS, and DISCUSSION

a. STRUCTURE.

1. MOJAVE TOXIN. The complete sequence of the basic subunit of Mojave toxin from the venom of the Mojave rattlesnake (Crotalus s. scutulatus) was determined by protein sequencing. The basic subunit of Mojave toxin and crotoxin isomers sequenced to date have identical primary structures, except at four positions. In three of these, at positions 33, 37, and 69 the Mojave toxin sequence is identical to one of the known crotoxin sequences. Only at position 65 is there a unique difference, with crotoxin having arginine and Mojave toxin having either proline or glycine. Sequencing of the isolated basic subunit cDNA of Mojave toxin had proline at position 65 (Dr. Leonard Smith, unpublished data). Experimental details for the protein sequencing may be found in the paper by Aird, Kruggel and Kaiser (1990).

2. CROTOXIN. The B-chain of the acidic subunit of crotoxin proved refractory to Edman degradation. When subjected to sequence analysis using tandem mass spectrophotometry, pyroglutamate was found at the amino-terminal end, even though earlier attempts to deblock with pyroglutamate aminopeptidase were unsuccessful. The B-chain contained 35 amino acids and showed 91% amino acid identity with the corresponding segment of Mojave toxin, a

homologous neurotoxin from Crotalus scutulatus scutulatus (Bieber et al., 1990). The sequence of the last 24 residues of the B-chain is consistent with that previously published (Aird et al., 1985) except at position 20, where Edman degradation gave glycine and mass spectrometry gave glutamic acid. Additional details on the determination of the B-chain sequence using mass spectrometry may be seen in the reference by Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser (1990).

3. BOTHROPS ASPER PHOSPHOLIPASES. A myotoxic, basic phospholipase A_2 (pI>9.5) with anticoagulant activity was purified from the venom of Bothrops asper, and its amino acid sequence determined by automated Edman degradation. It is distinct from the B. asper phospholipase A_2 known as myotoxin I (Lomonte and Gutierrez, 1989), but cross-reacts with myotoxin I rabbit antisera, suggesting that the proteins are closely related isoforms. We have called it myotoxin III. To our knowledge, this is the first myotoxic phospholipase to be sequenced that lacks presynaptic neurotoxicity (i.v. LD_{50} = 8 μg/g in mice). The protein appears to exist as a monomer, contains 122 amino acids and fits with subgroup IIA of other sequenced phospholipase A_2 molecules. Its primary sequence shows greatest identity with ammodytoxin B (67%), a phospholipase A_2 presynaptic neurotoxin from Vipera ammodytes ammodytes venom. Hydropathy profiles of B. asper phospholipase and the ammodytoxins also show great similarities In contrast, even though the amino acid sequence identities between B. asper phospholipase and the basic subunit of crotoxin remain high (64%), their hydropathy profiles differ substantially. Domains and residues that may be responsible for neurotoxicity are discussed. Experimental details may be found in the reference of Kaiser, Gutierrez, Plummer, Aird, and Odell (1990)

4. BOTHROPS ASPER PHOSPHOLIPASE-LIKE MOLECULES. A second phospholipase-like molecule from the venom of B. asper was also sequenced. This protein, a basic dimeric myotoxic protein, myotoxin II, has a similar molecular weight and is immunologically cross-reactive with antibodies raised to previously isolated B. asper phospholipases A_2, except that it shows only 0.1% of the phospholipase activity against L-α-phosphatidylcholine in the

presence of Triton X-100. Its 121 amino acid sequence, determined by automated Edman degradation, clearly identifies it as a Lys-49 phospholipase A_2. Key amino acid differences between myotoxin II and phospholipase active proteins in the Ca^{++}-binding loop region, include Lys for Asp-49, Asp for Try-28, and Leu for Gly-32. The latter substitution has not previously been seen in Lys-49 proteins. Other substitutions near the amino terminus (Leu for Phe-5 and Gln for several different amino acids at position 11) may prove useful for identifying other Lys-49 proteins in viperid and crotalid venoms. Myotoxin II shows greater sequence identity with other Lys-49 proteins from different snake venoms (Agkistrodon piscivorous piscivorous, Bothrops atrox and Trimeresurus flavoviridis) than with another phospholipase A_2 active Asp-49 molecule isolated from the same B. asper venom. This work demonstrates that phospholipase activity per se, is not required in phospholipase molecules for either myotoxicity or edema inducing activities. Experimental details may be found in the manuscript by Francis, Gutierrez, Lomonte, and Kaiser (1991).

5. ATTEMPTS TO ENHANCE TOXICITY IN BASIC PHOSPHOLIPASES A_2 WITH THE ADDITION OF THE ACIDIC SUBUNIT OF CROTOXIN. We have examined the ability of the acidic subunit of crotoxin to synergistically enhance the toxicity of two different basic phospholipases A_2. These have included myotoxin III from B. asper and V8 phospholipase A_2 from Vipera russellii (provided by John Middlebrook, USAMRIID). In neither case was an enhancement in toxin lethality observed. With V8 phospholipase alone we determined an i.v. LD_{50} of 3.2 μg/g. Addition of an equal molar amount of the acidic subunit to the PLA_2 before injection, did not shorten the time to death in animals injected with 3.2 μg/g of PLA_2. Animals injected with 2.2 μg/g of PLA_2 plus an equal molar amount of the acidic subunit survived. We concluded that if association of the PLA_2 and the acidic subunit occurred, it does not increase toxicity of the PLA_2. Similar results using a similar experimental protocol were obtained with myotoxin III.

6. ATTEMPT TO ENHANCE TOXICITY OF THE BASIC SUBUNIT OF MOJAVE TOXIN WITH THE ACIDIC SUBUNIT (CbI) FROM THE NEUROTOXIN

COMPLEX FROM PSEUDOCERASTES FIELDI VENOM. In a complementary experiment, we attempted to synergistically enhance the toxicity of the basic subunit of Mojave toxin. Purified Cbl protein was kindly provided by Dr. Aviner Bdolah (Dept. of Zoology, George S. Wise Faculty of Life Sciences, Ramat Aviv, 69 978 Tel Aviv, Israel). Basic subunit of Mojave toxin was injected i.v. into female mice at near its LD_{50}-value (0.5 μg/g; 0.036 nmole/g). Basic subunit was also pre-mixed with Cbl subunit as well as the acidic subunit of Mojave toxin, 30 min before injection. Acidic subunit of Mojave toxin clearly potentiated the toxicity of the basic subunit (4 deaths in 4 animals within 60 min). Cbl did not (0 deaths in 4 animals after 24 hrs). Mojave toxin basic subunit alone at 0.5 μg/g caused death in 2 of 7 animals after 24 hrs, whereas Cbl at 2.5 μg/g caused no deaths in four animals. Cbl and Mojave toxin acidic subunit concentrations were both 2.5 μg/g and were present in 5 to 8-fold molar excess over the basic subunit in the mixtures. No enhancement in toxicity of the basic subunit of Mojave toxin was observed by the addition of the acidic Cbl subunit.

7. NOTECHIS. Venoms from Notechis scutatus scutatus, Notechis ater serventyi, Notechis ater humphreysi, and Notechis ater ater were compared using gel filtration, polyacrylamide gel electrophoresis, and western blotting. All venoms gave slightly different elution profiles on a Superose 12 gel filtration column. When examined by SDS-PAGE and western blotting, all venoms appeared to have notexin like proteins except for N. a. serventyi. N. a. serventyi lacked proteins migrating in the notexin molecular weight range, showed no reactivity toward a monoclonal antibody that reacted strongly with notexin in western blot analysis, and had twice the LD_{50}-value of the other three venoms. Experimental details may be found in the paper by John and Kaiser (1990).

8. NEW TOXINS (SCUTOXINS) FROM THE VENOM OF THE COMMON TIGER SNAKE (NOTECHIS SCUTATUS SCUTATUS). During our preparation of notexin and notechis II-5 under contract DAMD 17-86-C-6061, we isolated a protein which was less basic than either notexin or notechis II-5, but was as toxic as notexin. These new toxins are described in a publication by Francis, John, Seebart and Kaiser (1991), which also provides an alternative procedure for the

isolation of highly purified notexin and notechis II-5. The new toxins, scutoxin A and B represent two isoforms of a new toxic protein from the venom of the Australian Tiger Snake, Notechis scutatus scutatus. Both isoforms, of apparent mol. wt. 13,000, are less basic that either notexin or notechis II-5. They both have similar i.v LD_{50}-values in mice of ca. 0.006 μg/g, and phospholipase activities of about 136 μmoles fatty acid released $min^{-1}mg^{-1}$ at 37° when acting on phosphatidyl choline in the presence of Triton X-100. Toxicities of the scutoxins are the same as notexin and about seven-times more potent than notechis II-5. ELISAs and western blot analyses indicate that the new toxins are immunologically similar to notexin and notechis II-5, with phospholipase activities falling between these latter two proteins. When crude venom is initially passed over a gel filtration column, each scutoxin isoform co-elutes in a different fraction with notexin. Gel filtration experiments using purified samples of notexin and scutoxin have failed to demonstrate any evidence for the formation of higher molecular weight protein complexes. Peptide mapping suggests the presence of five glutamate residues in one of the protein isoforms. These findings, together with the high toxicity and active phospholipase levels, demonstrate that the new proteins are not the previously reported non-toxic and enzymatically inactive notechis II-1.

Recent unpublished sequencing results in collaboration with Dr. Jim Schmidt (USAMRIID), indicate that scutoxin A is identical to notexin except for two amino acid replacements. Scutoxin A contains an Arg at position 16, whereas notexin contains a Lys; scutoxin A contains Glu at position 82 and notexin a Lys. Scutoxin B has been sequenced through residue 112. Up to this point, the structures of scutoxins A and B are identical. Sequencing of the remaining C-terminal peptide of scutoxin B is in progress. When completed the work will be prepared for publication. These results clearly identify these toxins as notexin isoforms.

Scutoxins A and B differ from the isoform of notexin isolated by Mollier et al. (1989) and identified as notechis N_s, which was shown to differ from notexin by a single amino acid substitution at position 16, where Arg replaced the Lys in notexin. N_s retains Lys at position 82, unlike the scutoxins.

The combination of gel filtration on Sephacryl S-200 and cation-exchange chromatography used to isolate the scutoxins also permits

recovery of notexin and notechis II-5 in high purity.

9. PROTEINS FROM TIGER SNAKE VENOM THAT CAUSE HYPOTENSION AND HEMORRHAGE. During our purification of neurotoxic proteins from crude venom of N. s. scutatus, we encountered a set of acidic proteins which were toxic when injected i.v. into mice at ≈1μg/g. These proteins, called $HT_{a\ to\ i}$ cause hypotension and hemorrhage in mice. They have apparent molecular weights in the 18-21,000 dalton range, i.v. LD_{50}-values between 0.5 and 1.5 μg/g, and no detectable phospholipase, arginine-ester, proteolytic or hemolytic activities. A polyclonal antibody raised against HT_g binds to other purified proteins suggesting that they are isoforms of the same protein. Many other elapid crude venoms contain proteins which recognize the polyclonal antibody raised against HT_g. Crotalid and viperid crude venoms do not recognize this antibody although some of their component proteins are known to exhibit hypotensive and hemorrhagic activities. A combination of gel-filtration on Sephacryl S-200, cation-exchange and anion-exchange chromatography allows isolation of the N. s. scutatus proteins in high purity. These are the first hypotension inducing proteins to be purified from an Australian elapid.

10. PROPERTIES OF POLYCLONAL ANTIBODIES RAISED TO POSTSYNAPTIC NEUROTOXIN NOTECHIS III-4 FROM NOTECHIS SCUTATUS SCUTATUS. When we developed our new scheme for the isolation of notexin from N. s. scutatus venom, we identified several basic proteins with apparent molecular weights of ≈11kD. We subsequently demonstrated that one of these proteins was notechis III-4, a "long" post-synaptic neurotoxin containing 73 amino acids that had previously been purified and sequenced (Eaker et al., 1976; Halpert et al., 1979). Polyclonal antibodies raised against purified notechis III-4 were shown to recognize both conformational and linear epitopes in notechis III-4, but only conformational epitopes in other N. s. scutatus venom proteins including notexin and notechis II-5. Notechis III-4 is markedly deficient in venom from N. s. scutatus collected near Lake Alexandrina in Australia, and absent from venoms of Notechis ater subspecies, crotalids, and viperids. Of six other elapid venoms screened, only Bungarus multicinctus and Pseudonaja textilis showed weak cross-reactivity. Reactive protein

species in these venoms include the long post-synaptic neurotoxins α-bungarotoxin and pseudonajatoxin b. Surprisingly the polyclonal antibodies were only weakly neutralizing against notechis III-4 lethality. Most antigenic regions of the long postsynaptic toxins appear to be variable or non-conserved regions. A new method for purification of notechis III-4 is also reported.

11. A CROTOXIN-LIKE PROTEIN FROM THE VENOM OF A SOUTH AMERICAN RATTLESNAKE (CROTALUS DURISSUS COLLILINEATUS). Earlier work initiated on our first contract suggested that the venom of Crotalus durissus collilineatus contained a potent toxin. However, published studies by Faure and Bon (1987) reported that C. d. collilineatus venom contained only small amounts of a crotoxin-like neurotoxin in its venom. Subsequent fractionation of three different lots of collilineatus venom in our laboratory demonstrated substantial amounts of a crotoxin-like protein in all three venoms. In fact, nearly 80% of the protein in one lot represented the crotoxin-like material, the highest percentage that we have observed in any rattlesnake venom to date. Many of its properties are similar to those of crotoxin, including its non-covalent heterodimeric structure, electrophoretic mobility on SDS-PAGE, isoelectric focusing properties, toxicity in mice, immunological reactivity, multiple isoforms, phospholipase activity, peptide map, and instability on an anion-exchange column. These results, reported in a manuscript by Lennon and Kaiser (1990), indicate that 'collilineatus toxin' is strongly homologous with crotoxin, found in the venom of C. d. terrificus, and all other characterized rattlesnake neurotoxins.

12. MOJAVE TOXIN SUBUNIT PREPARATION. We completed our preparation of highly purified acidic and basic subunits (20 mg each) for Dr. Keith Ward (Naval Research Laboratories, Washington, D.C.) using the published procedure of Aird and Kaiser (1985). Ward's laboratory has been involved in attempts at their crystallization, but has not been successful in obtaining usable crystals.

13. CROTOXIN SUBUNIT CROSS-LINKING. In work initiated under the last contract and recently completed, crotoxin was cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydro-

chloride. Cross-linked crotoxin had the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and 80% reduction of reactable lysine residues. It was also non-toxic, had reduced immunological cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crotoxin and had lost >95% of its phospholipase activity. Loss of toxicity was due to either subunit cross-linking or the modification of essential residues. See the paper by Lennon, Plummer, and Kaiser (1990) for details.

14. X-RAY CRYSTALLOGRAPHY OF MOJAVE TOXIN. Single crystals of intact Mojave toxin suitable for x-ray analysis have been prepared by Dr. Keith Ward (Lab for the Structure of Matter, Naval Research Laboratory, Washington, D.C.) from purified material we provided to him. Crystals are orthorhombic, exhibit the symmetry of space group $P2_12_12_1$, and have lattice constraints: a=38.6, b=69.9, and c=77.6 Å. Over 11,000 items of reflection data have been collected to a resolution of 2.2 Å. The data have been phased by the molecular replacement method using as a model the structure of dimeric PLA_2 from C. atrox. Details of the region of the unit cell containing the basic, phospholipase subunit is well resolved, but the region containing the acidic, chaperone subunit is difficult to interpret and model building in this region is tenuous. Despite extensive computational efforts using several different refinement programs, including molecular dynamics, the crystallographic R-value remains at 0.30. This structure represents the first presynaptic neurotoxin to be reported using x-ray diffraction methods (Collins et al., 1989; Ward and Pattabiraman, 1990). While improvements in the phase information would be desirable, the unit cell containing the basic subunit is well resolved and the coordinates are available for modeling and energy minimization studies. At the very least, we should be able to verify the disulfide bond arrangement is the basic subunit of crotoxin from the existing data.

In addition to samples of acidic and basic subunit of Mojave toxin, 5-15 mg samples of ammodytoxin, caudoxin, taipoxin, and paradoxin have been subjected to crystallization trials by Ward. Screens with ammonium sulfate, polyethylene glycol, and phosphate as precipitating agents have not resulted in crystals large enough for characterization by crystallographic methods.

b. MECHANISM OF ACTION.

1. CROTOXIN IODINATION AND BINDING STUDIES. Crotoxin has been iodinated using the chloramine-T method with both ^{125}I, and ^{127}I, and extensively characterized. We obtain from 0.4-0.9 mol of ^{125}I per mol of intact crotoxin with a distribution of label between the basic:acidic subunits of 4:1. LD_{50}-values (i.v.) of ^{127}I-labeled crotoxin and sham-reacted crotoxin were 0.060 and 0.062 μg/g in mice, respectively, with control crotoxin LD_{50}-values of 0.045μg/g. Phospholipase activity for native intact, native basic, ^{127}I-labeled, and mock-labeled crotoxin were determined to be 24, 85, 81, and 60 μmoles of L-α-phosphatidylcholine hydrolyzed min^{-1} mg^{-1}. In solid phase ELISAs, native, ^{127}I-labeled, and mock-labeled crotoxin all competed with biotinylated intact crotoxin for sites on each of the plate-bound monoclonal antibodies to crotoxin in a similar manner. Based on these studies, we feel that the iodinated crotoxin is similar enough to unmodified crotoxin to use in binding experiments.

Crotoxin, iodinated as described above was used to demonstrate high affinity, specific binding to guinea-pig (Cavia porcellus) brain synaptosomes and synaptosomal membrane fragments. ^{125}I-crotoxin binding to the membrane fragments displays two binding plateaus, (K_{d1}= 4 nM and K_{d2} = 87 nM, B_{max1}= 2, and B_{max2} =4 pmoles/mg membrane protein), but binding to whole synaptosomes revealed only one plateau (K_d = 2 nM and B_{max} = 5 pmoles/mg membrane protein). Rosenthal analysis of Scatchard plots yielded similar binding constants in the absence or presence of 0.025% Triton X-100. In addition to equilibrium analysis, kinetic analyses of ^{125}I-crotoxin binding to synaptosomal membrane fragments gave a K_d-value of 3 nM. The K_d value was not significantly changed by the exclusion of added calcium, but the binding site number was lowered. Crotoxin binding was inhibited by the acidic subunit of crotoxin and several presynaptic neurotoxins, which were classified according to their inhibitory properties as, strong (acidic subunit of crotoxin, Mojave toxin, concolor toxin, taipoxin, and pseudexin), moderate (ammodytoxin A and textilotoxin), weak (notexin and scutoxin A), very weak (notechis II-5) and non-inhibitory (basic subunit of crotoxin, β-bungarotoxin, Crotalus atrox and porcine pancreatic

phospholipases A_2, dendrotoxin, and notechis III-4). Purified acidic subunit of crotoxin, the most potent competitor of crotoxin binding, was somewhat more competitive than intact crotoxin and other strong inhibitors on a molar basis. Strong, moderate and weak inhibitor groups each differed from the preceding group by requiring about a ten fold increase in concentration to effect 50% inhibition of crotoxin binding. The weak group was therefore at least two-orders of magnitude less effective than the strong inhibition shown by the acidic subunit of crotoxin. Treatment of synaptosomal membranes with protease K lowered ^{125}I- crotoxin binding, whereas treatment with trypsin did not. Iodinated, phospholipase A_2 from C. atrox venom showed no specific binding to whole synaptosomes. Our findings demonstrate the presence and describe some of the properties of high affinity, specific binding sites in brain tissue for crotoxin and related presynaptic neurotoxins. Details of this work have been described in publications by Degn, Seebart, Plummer, and Kaiser (1990) and Degn, Seebart, and Kaiser (1991).

2. CROSS-LINKING EXPERIMENTS. As a continuation and extension of Dr. Laura Degn's binding studies of ^{125}I-crotoxin to synaptosomes and synaptic membranes, she completed initial experiments involving both chemical and photoaffinity cross-linking of ^{125}I-crotoxin to synaptosomal membranes. Chemical cross-linking with disuccinimidyl suberate (DSS) yielded a 53kD-labeled protein separated by SDS-PAGE--assumed to be ≈39kD membrane protein after subtracting 14kD for the molecular weight of the basic subunit of crotoxin. Addition of DTT to the reaction product before electrophoresis had no effect on its mobility. Further, no cross-linking was observed with erythrocytes or when ^{125}I-phospholipase A_2 from C. atrox was substituted for ^{125}I-crotoxin. Photoaffinity labeling of ^{125}I-crotoxin-N-hydroxysuccinimidyl-4-azidobenzoate (HSAB), to synaptosomes and synaptosomal membranes yielded bands on SDS-PAGE migrating at 102kD, equating to a 88kD protein with the molecular weight of the 14kD basic subunit subtracted. In two different experiments, various inhibitors added to a final concentration of 2×10^{-7} M were added to membranes and ^{125}I-crotoxin-HSAB. Proteins not competing with the photolabeling included porcine pancreatic phospholipase A_2, C. atrox Phospholipase

A_2, β-bungarotoxin, and dendrotoxin. Inhibitors included pseudexin, taipoxin, paradoxin, textilotoxin, ammodytoxin, notexin, concolor toxin, and both subunits of crotoxin.

3. PHARMACOLOGICAL EXPERIMENTS. In collaboration with Dr. Lance Simpson (Jefferson Medical College, Philadelphia), we have been involved in pharmacological experiments involving phospholipase A_2 presynaptic neurotoxins from snake venoms, their subunits, and non-neurotoxin phospholipases A_2. This work has been designed to gain greater insight into the sequence of events in phospholipase A_2 toxin-induced blockage of neurotransmitter release. Two series of experiments have been completed to date. One series is described in the manuscript by Trivedi, Kaiser, Tanaka, and Simpson (1989). In this study, crotoxin and its two subunits were tested for their neuromuscular blocking activity on the phrenic nerve-hemidiaphragm preparation. Two types of experimental paradigms were used, the first of which separated the toxin binding step from subsequent events in paralysis and the second of which did not. In both paradigms the toxin produced concentration-dependent blockade of transmission. However, the results with low concentrations were variable, and in some cases complete neuromuscular blockade did not develop. The isolated acidic and basic subunits possessed little toxicity. In experiments designed to characterize binding, the intact toxin displayed the following properties: 1) the apparent half-time for tissue association was about 22 min; 2) binding was not affected by low temperature, the presence or absence of nerve stimulation and the substitution of strontium for calcium; and 3) when binding was allowed to go to completion, reversibility was negligible. Pretreatment of tissues with isolated subunits of crotoxin did not enhance or inhibit the binding of the parent molecule. Modification of one histidine residue in the isolated basic subunit, followed by reconstitution with unmodified toxin acidic subunit, generated a molecule that possessed only about 10% of the neurotoxicity of the native toxin. The modified toxin could not be used to antagonize binding of the native toxin. Both polyclonal and monoclonal antibodies were generated that separated the binding step from later events in paralysis, the polyclonal preparation continued to locate and partially neutralize tissue-bound toxin. In experiments that

initiated events that follow binding, polyclonal antibodies were progressively less effective with time in neutralizing toxin. The monoclonal preparation did not neutralize toxin after the binding step was complete.

In a second series of pharmacological and physical chemistry experiments, attempts were made tc identify the site at which phospholipase A_2 neurotoxins localize to exert their poisoning effects. Experiments were conducted on five phospholipase A_2 neurotoxins of differing chain structures and antigenicities [notexin (one chain); crotoxin (two chains not covalently bound), β-bungarotoxin (two chains covalently bound); taipoxin (three chains), and textilotoxin (five chains; one copy each of three chains and two copies of a fourth chain)]. Comparison experiments were done with three clostridial neurotoxins (botulinum neurotoxin types A and B, and tetanus toxin). Phospholipase A_2 neurotoxins produced concentration-dependent blockade of neuromuscular transmission. There was no obvious relationship between chain structure and potency, but there was an indication of a relationship between chain structure and binding. The binding of notexin was substantially reversible, the binding of crotoxin was slightly reversible, and the binding of β-bungarotoxin, taipoxin and textilotoxin was poorly reversible. Experiments with neutralizing antibodies indicated that phospholipase A_2 neurotoxins became associated with receptors on or near the cell surface. This binding did not produce neuromuscular blockade. When exposed to physiological temperatures and nerve stimulation, receptor-bound toxin disappeared from accessibility to neutralizing antibody. This finding suggests that there was some form of molecular rearrangement. The two most likely possibilities are: 1) there was a change in the conformation of the toxin molecule, or 2) there was a change in the relationship between the toxin and the membrane. The molecular rearrangement step did not produce neuromuscular blockage. At a later time there was onset of paralysis; the amount of time necessary for onset of blockade was a function of toxin concentration. Phospholipase A_2 neurotoxins were not antagonized by drugs that inhibit receptor-mediated endocytosis. In addition, phospholipase A_2 neurotoxins did not display the pH-induced conformational changes that are typical of other

endocytosed proteins, such as clostridial neurotoxins. However, phospholipase A_2 neurotoxins were antagonized by strontium, and this antagonism was expressed against toxins that were free in solution and toxins that were bound to the cell surface. Limited antagonism was expressed after toxins had undergone molecular rearrangement, and no antagonism was expressed after toxin-induced neuromuscular blockade. The cumulative data suggest that phospholipase A_2 neurotoxins are not internalized to produce their poisoning effects. These toxins appear to act on he plasma membrane, and this is the site at which they initiate the events that culminate in neuromuscular blockade.

The pharmacological collaborative studies with Simpson's laboratory are continuing. We have largely been providing his research group with purified phospholipase A_2 neurotoxins.

4. ELECTRON MICROSCOPY STUDIES. To complement the pharmacological experiments described above, we have been collaborating with Dr. Robert Jenkins (Department of Zoology and Physiology, University of Wyoming), an electron microscopist, who is conducting experiments designed to look at membrane labeling on mouse diaphragms from animals treated with crotoxin.

Experiments preceding the EM work, employed autoradiography of whole diaphragms of mice injected with ^{125}I-crotoxin and ^{125}I-notechis III-4 (a postsynaptic toxin) for a control. One mouse was injected i.v. with 2 LD_{50}-values of ^{125}I-crotoxin and sacrificed 40 min later. Another received 1.3 μg of ^{125}I-notechis III-4 ($LD_{50} \approx 0.15$ μg/g), and was also sacrificed 40 min later. After perfusion, the diaphragm was dissected, fixed, and dried overnight in a stretched position, and exposed to Kodak diagnostic film SB the following day for three hours. The autoradiogram for ^{125}I-crotoxin showed a diffuse pattern. (In another time-study experiment, where mice were sacrificed at 5, 10, 20, 40, and 80 min after the 2 LD_{50} injection, essentially the same results were seen). ^{125}I-Notechis III-4 produced a pattern of radiation which clearly corresponds to neuromuscular junction areas, which can also be stained specifically for acetylcholine esterase (Rash and Ellisman, 1974). Li and Tseng (1966) showed a number of years ago that β-bungarotoxin also produced a diffuse pattern of labeling in the diaphragm when

administered in a similar way and that α-bungarotoxin delineates the neuromuscuiar junctions, consistent with our results. Why the presynaptic neurotoxins give the diffuse pattern is unknown.

Jenkins' laboratory has developed EM methodologies and has examined crotoxin distribution in treated mice sacrificed at different time intervals following intoxication. Animals have been sacrificed, tissues fixed and embedded, and treated with murine monoclonal antibodies to crotoxin, followed by anti-mouse antibodies conjugated to gold beads. We are hopeful that results from such studies will complement the pharmacological experiments in progress in Simpson's laboratory.

5. IN VITRO SYSTEM FOR EXAMINING RESPONSE TO PRESYNAPTIC NEUROTOXINS. During the course of this work we spent well over a year attempting to apply bioluminescence techniques to monitor the continuous detection of acetylcholine from guinea pig brain synaptosomes. In theory, the method described by Israel and Lesbats (1987) should permit measurement of acetylcholine on a continuous basis. We were unsuccessful in setting up this system in our laboratory. With brain synaptosomes the background or "spontaneous leakage" of acetylcholine is significantly high to give a high background signal relative to the signal generated during e.g. the triggering by KCl depolarization. Thus, with brain synaptosomes we were unable to successfully use bioluminescence methodology.

c. IMMUNOLOGY.

1. MONOCLONAL ANTIBODY CHARACTERIZATION AND SYNTHETIC PEPTIDE DESIGN. We have completed our examination of cross-reactivity of our four monoclonal antibodies and rabbit polyclonal antibodies raised against the basic subunit of crotoxin. Some of this work was described in my Final Report for contract no. DAMD 17-86-C-6061.

All four monoclonal antibodies appear to react with unique epitopes. Western blots on both non-reduced and reduced crotoxin samples, suggested that only one of the monoclonal antibodies (#11) partially reacted with reduced crotoxin. This indicated that it might at least partially recognize a continuous epitope. Octapeptides homologous with the complete sequence of the basic subunit of

crotoxin were carried out on polyethylene pens according to the procedure of Geysen et al. (1987). These overlapping peptides were then used to screen our four monoclonal antibodies in an attempt to determine their antigenic determinants. Multiple assays failed to give consistent results, indicating that none of the four monoclonal antibodies recognized our solid-phase, octapeptides. The same octapeptides were used to screen rabbit polyclonal antibodies. From the polyclonal assays, we identified four region from the basic subunit sequence that appeared antigenic. These were sequences 113-122 (SRSRGPSETC), peptide A; 102-115 (TYKYGYMFYPDSRC), peptide B; 31-44 (GGRGRPKDATDRSC), peptide C; and 1-9 (HLLQFNKMIC), peptide D. The highlighted S's in the 113-122 and 31-44 peptides have been substituted for a C. See Fig. 1 for the complete sequence of crotoxin subunits. These peptides were synthesized and coupled to carrier proteins (rabbit serum albumin) through their carboxyl-terminus sulfhydryl residues which naturally occurred in that position in peptides A, B, and C. An additional C was added to the C-terminus in peptide D for coupling purposes. Rabbits were immunized with conjugated proteins, boosted three times, and their antisera assayed for reactive antibodies.

2. ELISAS. Enzyme-linked immunosorbant assays (ELISAS) were carried out to determine the reactivity of polyclonal antisera raised against the four synthetic peptides conjugated to rabbit serum albumin. In addition, anti-peptide sera were screened for reactivity against free synthetic peptides, intact crotoxin, as well as its subunits.

Titers ranging from 1:250 to 1:160,000 were determined for the peptide antisera when assayed against their homologous, purified peptides conjugated to rabbit serum albumin (Fig 2). None of the antisera reacted with rabbit serum albumin alone and only two sera, from rabbits immunized with peptide B, showed reactivity when run against free homologous, non-conjugated peptide bound to the ELiSA plate (not shown). Plate affinity for the free peptides may be a problem in some experiments, since in an assay using intact crotoxin antisera against both free and conjugated peptides, the conjugated peptides gave substantially better reactivity (not shown).

Antipeptide sera raised against three of the four peptides reacted well with intact crotoxin and its basic subunit (Fig. 3). Peptide D

reacted most strongly in both assays, while C showed better reactivity than B. Peptide A did not react with either intact crotoxin or its basic subunit. None of the antipeptide sera reacted with the acidic subunit of crotoxin. This was not unexpected since the antigenic regions of the basic subunit identified by the Geysen procedure were either missing in the acidic subunit or had substantial amino acid sequence differences. For example, as shown in Fig. 1, peptide A had five amino acid differences out of nine: B had six amino acids missing in the corresponding acidic chain and six of eight of the remaining amino acids differed; peptide C was the most similar, with only four differences out of 14; and the entire peptide D region was absent in the acidic chain.

3. TOXIN NEUTRALIZATION WITH PEPTIDE ANTISERA. Antisera raised against each synthetic peptide were individually pre-mixed with 2 LD_{50}-doses of intact crotoxin before injection into mice. Neutralizing polyclonal antisera raised to intact crotoxin was combined with intact crotoxin in the same proportions and injected, serving as a positive control. A mixture of four antisera raised against each of the four different peptides was also pre-mixed with intact crotoxin and injected into mice. Only polyclonal antisera raised to intact crotoxin totally neutralized the intact crotoxin. Antipeptide sera raised against peptides B2 and B4 did delay deaths of mice 1.5 to 2 times that of mice injected with intact crotoxin alone (see Table 1). Combined antisera pre-mixed with crotoxin also appeared to delay death in animals relative to controls (see Table 1).

4. F_{ab} FRAGMENTS. One of our aims in this contract was to prepare F_{ab} fragments of our neutralizing monoclonal antibody for crystallization attempts of the F_{ab}-antigen complex by Keith Ward. Ward obtained 14 mg crude monoclonal antibody directly from Dr. John Middlebrook (USAMRIID) and prepared F_{ab} fragments in his laboratory. Some difficulty occurred in achieving complete digestion without long incubation times. Long incubation times produced a wide array of digested species, which then had to be purified prior to crystal growth experiments. Total digest was initially purified by protein A affinity chromatography, which separated the F_{ab} fragments from the F_c region. Anion-exchange

column chromatography was used to purify the different species of F_{ab} fragments produced from the digestion experiments. There were four major F_{ab} fragments with pI's of 5.3, 5.9, 6.5, and 7.2, all of which retained their ability to bind Mojave toxin as determined by dot blot ELISAs. Complete purification of all four fragments was never completely attained. One of these fragments was hyperpurified to the point where it was >98% pure as determined by isoelectric focusing, and amounted to 1.2 mg of material. Crystal growth experiments conducted with this small sample have not been successful. In view of the difficulties that Ward's laboratory has had with determining the crystal structure of intact Mojave toxin, it is probably premature to pursue additional studies on the crystallization of the F_{ab}-antigen complex. Technically, by adjusting proteolytic digestion times to increase yields followed by improving the degree of purification of the F_{ab} samples by preparative isoelectric focusing or related technique, it should be possible to achieve the purification necessary to achieve F_{ab}-crotoxin complex crystallization.

5. NATURAL RESISTANCE TO SNAKE VENOMS. Certain mammals and especially snakes are resistant to high doses of snake venom. An undergraduate in my laboratory has been working on the isolation of an antihemorrhagic factor present in the blood sera of the western diamondback rattlesnake (C. atrox). Using a combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography, he has successfully isolated a group of closely related acidic proteins (isoforms?) with apparent molecular weights of ≈57 kD and pI-values ≈5.9. When mixed with crude C. atrox venom, the purified preparation inhibits the normal hemorrhagic activity shown by the crude venom in mice. In vitro the antihemorrhagic preparation inhibits the proteolytic activities of hemorrhagic toxin A from C. atrox and a purified hemorrhagic toxin from C. v. viridis, toward fibrinogen. The antihemorrhagic preparation also forms a stable complex with unidentified proteins when mixed with crude venoms from a variety of snakes as demonstrated by electrophoresis on non-denaturing polyacrylamide gels. Partial sequencing of the amino-terminal region of the purified antihemorrhagic factor gave the sequence FQLAG NMDVN TKGTK DWADI G. No reasonable matches were found in a protein

sequence database search. Our isolated factor is probably similar to that recently described by Weissenberg et al. (1991). We are preparing a manuscript for publication.

In keeping with our interests on natural mechanisms of snake venom resistance, we have been involved in a collaborative study with Dr. George Odell (Department of Biochemistry, Oklahoma State University, Stillwater) describing the presence of large amounts of citrate in some snake venoms (Freitas et al., 1992). We determined citrate concentrations in different viper, crotalid, and elapid venoms. In Bothrops asper venom e.g., we estimated that citrate concentrations were about 95 mM. Calcium-ion concentrations varied from 2.5 to 3.6 mM, suggesting that the high relative citrate level may serve to chelate endogenous divalent metal cations, thereby inactivating divalent cation requiring enzymes. Control experiments with purified B. asper phospholipase A_2 (myotoxin III), in the presence of 2.5 mM Ca^{++}, showed that the enzyme was completely inhibited by 20 mM citrate. Other divalent-metal requiring enzymes such as 5'-nucleotidase and phosphodiesterase are also partially inhibited by citrate. By forming complexes with divalent metal ions, citrate reduces the activities of selected enzymes in snake venoms. Secretion of high concentrations of citrate may represent an important mechanism by which snakes protect themselves against the toxic effects of their own venoms (Francis et al., 1992).

d. MOLECULAR BIOLOGY.

1. CLONING. Messenger RNA was originally isolated from the venom gland of C. d. terrificus, and it was reverse transcribed. The product was amplified using the polymerase chain reaction and cloned into Bluescript II KS plasmids. Clones with inserts were partially sequenced, using the double-stranded method. All isolated clones were acidic subunit clones and of the six that were partially sequenced, all showed substantial sequence variability. It was unclear whether these represented different genes or whether we inadvertently introduced these base changes during the amplification reaction. We decided to terminate our work on the C. d. terrificus materials and focus on cDNA and genomic libraries from the tissue of the Mojave rattlesnake (C. s. scutulatus), in

collaboration with Dr. Leonard Smith (USAMRIID). Smith's laboratory has prepared the cDNA library, screened it for acidic and basic subunit genes, isolated several clones, and has completed sequencing three acidic subunit and four basic subunit clones. Sequences of two of these clones, one of the acidic subunit and one for the basic are shown in Fig. 4A and B Both clones contain an identical leader sequence which codes for 16 amino acids. In the basic subunit this leader sequence immediately precedes the amino-terminal end of the mature protein, so that cleavage of the initial translation product between Gly-His generates the amino-terminal end of the mature basic subunit (Fig. 5). The acidic subunit proteins appears to be made as a pre-pro-protein. Not only must the 16 amino acid leader sequence be removed to yield the pro-protein, but three additional peptides must be removed to generate the mature three peptides that make up the acidic subunit. This proteolytic processing probably occurs after formation of the seven disulfide bonds found in the subunit, and results from a combined action of endo- and exoproteases. Virtually nothing is known about these processing events.

Sequencing of the basic subunit cDNA of Mojave toxin by L. Smith, gave the same amino acid sequence which we found earlier for the basic subunit by protein sequencing (Aird, Kruggel, and Kaiser, 1990). Smith found proline at position 65 in this clone and we found a mixture of proline/glycine at this position in the protein.

The cDNA sequence determined by Smith for the acidic subunit of Mojave toxin corresponds exactly with the corresponding protein sequences found by Bieber et al. (1989), from protein sequencing. Position 68 (Fig. 5) is encoded as glutamine, which cyclizes to form pyroglutamate at the amino-terminal end of the B-peptide in the mature acidic subunit.

My laboratory is developing a strategy to express these cDNA genes in an E. coli system. To date most of our work has been with the basic subunit. We inserted the cDNA clone for the basic subunit into an expression vector pTM-N (kindly provided by Dr. T. Deng). This vector is derived from the pET system developed for the cloning and expression of proteins and described in detail by Studier et al. (1990). The vector has a T7 gene 10 promoter, a Shine-Dalgarno sequence which is followed by the ompA sequence, and a multiple cloning site (MCS); in which we inserted our cDNA. Details of the

construction are shown in Fig. 6 below. T7 polymerase is present in the host bacterial chromosome under lac UV5 control and is inducible by the addition of IPTG to the culture media. T7 RNA polymerase is quite selective and active so that once induced a substantial proportion of the cell's resources are converted to target gene expression.

Our plasmid construction strategy was to insert the basic subunit of Mojave toxin cDNA into the plasmid to express and isolate the intact basic subunit. The construction shown in Fig. 6 should permit us to do this, since the basic subunit protein should be translated with the ompA signal peptide (21 amino acids) and linker peptide (6 amino acids) attached to the amino-terminal end, giving a transcript 149 amino acids long. The linker peptide contains the tetra peptide Ile-Glu-Gly-Arg, immediately ahead of the basic subunit protein's N-terminal His, which is recognized by the proteinase Factor Xa. Thus, isolated expressed protein, when treated with Factor Xa, should release the basic subunit of 122 amino acids.

This expression construct of the cDNA for the basic subunit of Mojave toxin has been sequenced. We have verified that the construct contained all features expected, including: (i) the Shine-Dalgarno sequence of the expressing vector, (ii) the E. coli ompA signal peptide coding region of the expression vector, (iii) the region coding for the proteolytic enzyme Factor Xa recognition site, and (iv) the sequence corresponding to the complete Mojave toxin basic subunit cDNA.

The pTM-N vector constructed as shown in Fig. 6 was used to transform competent E. coli HMS174 cells. About 100 transformants were probed with a labelled oligomer complementary to the 3'-end of the basic subunit cDNA. Six positive clones were picked, grown, plasmids isolated and cut with a combination of Eco RI/NcoI. Five of the six clones had an insert of the expected ca. 450 bp. One was selected and used to transform four different competent E. coli strains obtained from Novagen (Madison, WI). There were the non-expression strain BL21 and three expression strains BL21(DE3), BL21(DE3)LysE, and BL21(DE3)LysS. Using the same concentration of plasmid, we obtained ≈100 transformants/plate with the BL21 strain and 15 transformants/plate with the LysE strain. No transformants were found with the other two expression strains, and the LysE transformants produced small colonies. Transformation controls with uncut pTM-N plasmid gave ≈200

colonies/plate and those with no DNA added gave none. This suggested that the constructed plasmid was being expressed, but that for the target gene to be stabilized and maintained it required the presence of endogenous T7 lysozyme--a natural inhibitor of T7 RNA polymerase. It stabilizes target plasmids by decreasing the basal activity of T7 polymerase, but it does not prevent induction of high levels of target proteins.

Transformed BL21(DE3)LysE containing the pTM-N plasmid with the basic subunit of Mojave toxin insert, identified as BL21(DE3)LysE-pIK-2, was grown in L-broth with shaking and induced with 1mM IPTG when turbidity at A_{600} reached 0.6. Aliquots removed at increasing time intervals (0 to 4 hours) were centrifuged, treated with protein solubilizing solution, heated to 90° for 10 min, and samples were run on 15% SDS-PAGE. Coomassie staining revealed increasing amounts of a protein doublet band with a molecular weight of 17-18 kD (Fig. 7A), that expected for a protein of 149 amino acids. This region also reacted positively in a western blot using primary polyclonal antibodies against Mojave toxin (Fig. 7B). Integration of zero and four hour sample lanes of Fig. 7A, indicated that 3-4% of the total protein was present in the doublet band, compare Fig. 7C and 7D. These results indicate that we are synthesizing the basic subunit in our expression system. Studies are in progress to better characterize the products, including the re-oxidation of the basic subunit and treatment with Factor Xa to generate mature length basic subunit protein.

Total genomic DNA was prepared from C. scutulatus, type A liver as described by Davis et al., 1986. A kit from Promega Biotech was used to construct the genomic library using the manufacturer's protocols. C. scutulatus genomic DNA was randomly digested with Sau 3AI, under conditions to generate mostly large (15-25 kb) DNA fragments. Overhanging ends of the insert DNA were then partially filled in with dGTP and dATP using Klenow fragment of DNA polymerase I. After initial reaction optimization, the appropriate ratio of insert and vector DNA were mixed, ligated using T4 DNA ligase, packaged into phage particles (lambda GEM-11 Xho half-site arms from Promega), and used to infect host strain E. coli KW-251. About 3.6×10^5 plaque forming units (pfu) were obtained which was

estimated to be a 95% representation of the total C. scutulatus genome. The library was amplified before screening with 42-base oligomeric DNA probes identical with the 5'-end of the basic subunit coding strand (5'-CACCTGCTGCAATTCAACAAGATGATCAAGTTTGAGA CAAGG-3') and the 5'-end of the acidic subunit A-chain coding strand (5'-GGATGCTACTGTGGCGCGGGGGGCCAAGGCTGGCCACAGGAC-3'). ABOUT 150,000 pfu were screened with the above 42-mer probes and putative acidic and basic subunit clones identified. Three rounds of screening resulted in the purification of eight λ-DNA clones, with three positive for the acidic subunit, five for the basic subunit, and two hybridizing with both subunit probes. Insert size ranged from 7 to 16 kb.

Hybridization oligonucleotides complementary to DNA sequences immediately 5' (primer #1: 5'-TGCATGCCTGCAGAGACTTA-3') and 3' (primer #2: 5-CCCAGGTCTGGATTGAGGAGG-3') to the coding regions of both subunits of Mojave toxin were synthesized, and were used as probes for subcloning. Preliminary hybridization experiments using the above probes suggested that five of the eight clones were truncated at the 5'- end of the toxin gene. Thus, we have one full-length putative acidic subunit gene clone, and two full-length putative basic subunit gene clones. Two of these three clones also hybridized with both of the 42-mer oligomers mentioned above.

Sequencing of the gene for Mojave toxin acidic subunit is now complete. The gene is composed of four exons (coding region) and three introns (non-coding). The first exon consists of the leader peptide for the gene product; the remaining three exons make up the "pre-acidic subunit", which is subsequently processed into three peptides. The DNA sequence of the coding regions correspond exactly with the cDNA sequence for Mojave toxin acidic subunit (unpublished data, Dr. Leonard Smith). Excluding the 5' and 3'-flanking regions, the gene spans 1615 base pairs. A portion of the acidic subunit gene which includes the four exons is shown in Fig. 8.

About 2500 bases "upstream" (5') to the acidic subunit gene, and on the opposite strand of the acidic subunit gene is a possible "pseudogene" which shows about 80 percent homology with the actual acidic subunit gene. Its sequence has not been completely determined.

Cloning and sequencing of the basic subunit of Mojave toxin gene is in progress.

BASIC SUBUNIT REOXIDATION. We have conducted a series of in vitro reduction and re-oxidation studies on the isolated basic subunit of Mojave toxin, in preparation for the oxidation and naturation of our expressed form of the basic subunit. Experiments using the procedure of Thannhauser and Scheraga (1985) and about another half dozen other published methods, have not been successful in restoring phospholipase activity to the reduced subunit. We did successfully restore about 10% of the lipase activity using the renaturation scheme of Kelley, Crowl, and Dennis (1992). We modified this procedure and have now recovered 30-40% of the enzymatic activity. Briefly, this procedure calls for dissolving the basic subunit in 8 M GuHCl to give 1 mg/ml solution and reducing overnight with 100 mM DTT. Then, DTT is removed by passing the reduced solution over Biogel P-6DG equilibrated with 50 mM Tris (pH 8.5), 5 mM EDTA, and 6 M GuHCl. Basic subunit is immediately diluted to 5-10 μg/ml in renaturation buffer containing 50 mM Tris-HCl (pH 8 or 8.5), 1 mM EDTA, and 0.2% Triton X-100, producing a final GuHCl concentration of 9-18 mM. Solid cysteine and $CaCl_2$ was added to give each a final concentration of 10 mM and the pH adjusted to 8.0 or 8.5. Activity does not seem to increase after 48 hours. Some refinement of this procedure in still being carried out.

2. IN VITRO CULTURING OF SNAKE VENOM GLAND CELLS FROM CROTALUS VIRIDIS CONCOLOR. We have been interested for some time in establishing a cell culture of snake venom gland cells. Earlier unpublished work by Middlebrook suggested that it might be possible to establish such a line. More recently, Sells et al. (1989) reported the establishment of a long-term venom gland cell culture from Bitis gabonica. In collaboration with Kruger Bryant, Director of an on-campus tissue culture laboratory, we attempted to establish a venom gland cell culture from a young C. v. concolor snake in June, 1990. These cells remained viable and dividing at a very slow rate for at least three months. We also initiated a second culture of venom gland tissues from an adult C. v. viridis in November, 1990 using slightly different procedures in an attempt to achieve a more rapidly dividing culture. Results similar to those obtained with concolor were achieved.

Development of a viable venom gland cell line is critical for future studies of crotoxin subunit processing, as well as other venom production processes. The acidic subunit, is e.g. composed of three peptides which appear to be generated from a higher molecular weight precursor by several proteolytic cleavages. Identification and characterization of these processing proteases will require a viable tissue culture source of venom gland cells for raw material.

CONCLUSIONS

We have completed the sequencing of the basic subunit of Mojave toxin and the B-chain of crotoxin. This work completes the amino acid sequence studies on these two proteins, except for the determination of the disulfide bond arrangements within each subunit of the rattlesnake neurotoxins. We initially proposed a chemical determination for these, but now feel that these can best be determined by analysis of the x-ray data on the basic subunit of Mojave toxin collected in collaboration with Dr. Keith Ward.

Amino acid sequences have been determined for two myotoxins from the venom of Bothrops asper. One, myotoxin III, a potent phospholipase A_2 with 67% sequence identity with ammodytoxin a, represents the first myotoxin phospholipase sequenced that lacks presynaptic neurotoxicity. Domains and residues that may be responsible for neurotoxicity are suggested. The second phospholipase-like molecule sequenced, myotoxin II is a lysine-49 phospholipase A_2 with key amino acid differences from active phospholipases and exhibits <0.1% of the phospholipase activity of myotoxin III. This work demonstrates that phospholipase activity per se is not required in phospholipase molecules for either myotoxicity or edema inducing activities.

Attempts to enhance the toxicity in normally non-toxic basic phospholipases A_2 from B. asper and V. russellii by the addition of the acidic subunit of crotoxin have been unsuccessful. Additionally, in one experiment with a non-toxic acidic subunit from the neurotoxic complex from P. fieldi venom, the lethality of Mojave toxin basic subunit was not potentiated. Bon et al. (1992) recently reported that the acidic subunit of crotoxin could enhance the lethal toxicity of the single chain β-neurotoxin agkistrodotoxin and modify its pharmacological effect on Torpedo synaptosomes. Sedimentation

experiments indicated that a heterocomplex was formed. There is 80% sequence identity between the basic subunit of crotoxin and agkistrodotoxin. This is the first report that I am aware of which demonstrates an enhancement in toxicity by mixing of subunits from different toxins, outside the closely related rattlesnake presynaptic neurotoxins. These findings clearly have some evolutionary implications. In our studies we only screened for toxicity enhancements and did not examine the mixture for formation of higher molecular weight heterocomplexes.

A number of interesting studies evolved from our purification of notexin from Notechis scutatus scutatus under contract DAMD 17-86-C-6061. One of these conclusively demonstrated that not all Notechis subspecies contained notexin or notexin-like proteins. Another study identified two isoforms of notexin (scutoxins), which appear to differ from notexin by at least two amino acid substitutions, but retain notexin's toxicity and phospholipase activity. A third examined the immunological properties of the postsynaptic neurotoxin notechis III-4 and cross-reactivity of a variety of venom proteins in Notechis and other venoms to antibodies raised against the long-chain neurotoxin. Finally, our identification of acidic proteins present in N. s. scutatus venom that exhibit both hypotensive and hemorrhagic activities. These are the first hypotension-inducing proteins isolated from an Australian elapid.

In contradiction to earlier reports in the literature (Faure and Bon, 1987), we found up to 80% of the protein present in the venom of Crotalus durissus collilineatus to consist of a crotoxin-like protein.

Chemical cross-linking of crotoxin subunits with a water-soluble carbodiimide results in a loss of immunological cross-reactivity, phospholipase activity, and toxicity. We could not determine whether this was the result of cross-linking directly or the modification of essential amino acid side chain residues.

X-ray crystallography studies of Mojave toxin in collaboration with Keith Ward have been disappointing. Despite considerable effort, the crystallographic R-value for Mojave toxin remains at 0.30. A value indicative of a rather ill-defined structure. The acidic subunit region of the dimeric structure was simply not well resolved.

Crotoxin iodination methodology has been developed and extensive binding studies have been carried out with the iodinated toxin on synaptosomes and their membranes. A high affinity (K_d= 2-4 nM),

specific binding site was identified for crotoxin that could be strongly competed for by Mojave toxin, concolor toxin, taipoxin, paradoxin, and pseudexin. Ammodytoxin a and textilotoxin were less competitive with crotoxin binding and notexin, scutoxin A, and notechis II-5 even less. Non-neurotoxic phospholipases A_2 from C. atrox and porcine pancreas showed little or no inhibition; nor did β-bungarotoxin, dendrotoxin, and the post-synaptic neurotoxin notechis III-4. Our binding and x-linking results suggest that crotoxin and several other presynaptic neurotoxins share common binding sites that may involve a protein on brain synaptosomal membranes.

A series of experiments with Dr. Lance Simpson, employing structurally different snake venom presynaptic neurotoxins, have been carried out on isolated mouse phrenic nerve-hemidiaphragm preparations. Using a number of different paradigms, the work has essentially shown that the binding of crotoxin to the preparation is distinct from the poisoning step. Also, unlike most potent, enzymatic toxins, phospholipase A_2 neurotoxins are not endocytosed to exert their effects. They appear to bind to specific receptors on the cell surface and then undergo a molecular rearrangement that gives them access to substrates in the membrane.

Electron microscopy experiments in collaboration with Dr. Robert Jenkins are in progress. These studies are examining crotoxin distribution in the phrenic nerve tissues at different time intervals after exposure to crotoxin. We are hopeful that these microscopy studies will complement the pharmacological experiments being carried out in Simpson's laboratory.

Studies designed to employ bioluminescence techniques to monitor continuous acetylcholine release from brain synaptosomes in vitro have been unsuccessful. Synaptosomes from brain tissues appear too "noisy" to use with this assay, apparently due to spontaneous acetylcholine release. Other biological properties of synaptosomes that could possibly be used to monitor effects that different toxins and other materials have on them, that could be used as a biological assay. One such property is choline uptake.

All four of our monoclonal antibodies raised against the basic subunit of crotoxin appear to react with unique, conformational epitopes. None of the four recognized any of the overlapping octapeptides, homologous with crotoxin's basic subunit sequence. In contrast, when rabbit polyclonal antibodies were screened in place of the monoclonals, four regions appeared antigenic; the two end sequences plus internal regions corresponding to residues 31-44 and 102-115. Four peptides corresponding to these antigenic regions

were synthesized, conjugated to rabbit serum albumin, and rabbits immunized. Resulting antisera failed to neutralize against crotoxin's lethality, although there was some extension of survival times (Table 1). In order to determine the epitopes of our monoclonal antibodies, we need to explore other available screening procedures. With the synthetic peptide antigens, we need to screen the literature for better carrier proteins.

Examination of various natural resistance mechanisms that animals have evolved to protect them from high doses of venom have potential medicinal and biochemical applications. In one study we have demonstrated that C. atrox snake sera contains a group of closely related acidic, glycoproteins that can form a tight complex with hemorrhagic venom toxins. This leads to their inactivation. In a second study, we have identified citrate as a major venom component of some venoms. At high citrate/divalent metal cation ratios found in venoms, complexes formed will reduce activities of divalent-metal requiring enzymes. Phospholipases A_2 are completely inhibited, whereas 5'-nucleotidase and phosphodiesterase are partially inhibited.

cDNA clones for the acidic and basic subunits of Mojave toxin have been generated and sequenced by Dr. Leonard Smith. He has kindly made these available to us. Sequences found in the cDNAs correlate well with those determined by protein sequencing. We have inserted the cDNA clone for the basic subunit into the pTM-N E. coli expression vector and demonstrated by SDS-PAGE and western blot analysis expression of the protein. A strategy for insertion of the acidic subunit cDNA clone into a similar expression vector has also been developed. In vitro reduction and re-oxidation studies on the isolated basic subunit of Mojave toxin have been carried out to determine optimum conditions for naturation of the expressed form of the basic subunit. Current conditions allow recovery of 30-40% of the enzymatic phospholipase activity associated with the basic subunit.

Cloning of the genomic genes for the acidic and basic subunit of Mojave toxin has been completed. Sequencing of the acidic gene is also complete. It is composed of four exons (coding region) and three introns (non-coding). The first exon consists of the leader peptide for the gene product; the remaining three exons make up the "pre-acidic subunit", which is subsequently processed into three peptides. The DNA sequence of the coding regions correspond exactly with the cDNA sequence for Mojave toxin acidic subunit (unpublished data, Dr. Leonard Smith). Excluding the 5'- and 3'-flanking regions,

the gene spans 1615 base pairs. About 2500 bases "upstream" (5') to the acidic subunit gene, and on the opposite strand of the acidic subunit gene is a possible pseudogene which shows about 80% homology with the actual acidic subunit gene. Its sequence has not been completely determined. Cloning and sequencing of the basic subunit gene of Mojave toxin is in progress.

RECOMMENDATIONS

1. Revise manuscripts that are currently in the publication pipeline and get them in press.
2. Examine the disulfide bond arrangements in Mojave toxin subunits from x-ray crystal structures now completed, and prepare a short publication in collaboration with Dr. Keith Ward describing our findings.
3. Complete the sequencing of scutoxin B in collaboration with Dr. James Schmidt (USAMRIID) and prepare a manuscript describing the sequencing of scutoxin A and B for publication.
4. Continue our collaboration with Dr. Robert Jenkins in the electron microscopy laboratory here at the University of Wyoming, in an attempt to further characterize crotoxin interactions with nerve terminals in vivo, and determine whether internalization is observed. If so, is internalization required for crotoxin to express its toxicity?
5. Continue collaborative efforts with Dr. Lance Simpson's laboratory on the pharmacology of presynaptic neurotoxins.
6. Initiate new biochemical experiments involving snake venom presynaptic neurotoxins, to (i) determine whether they become chemically modified in vivo and what the functional effects of these, and other in vitro modifications, are; (ii) better characterize their carbohydrate moieties; (iii) examine higher-ordered structure using 2-D and 3-D NMR approaches; and (iv) examine their functional effects on endothelial cells in culture.
7. Summarize our collaborative work with Dr. Leonard Smith (USAMRIID) on the isolation, cloning, and sequencing of the cDNAs and genes for the subunits of Mojave toxin and prepare the material for publication. Continue to develop the E. coli expression system for ultimately generating non-toxic, immunogenic forms of the basic subunit for immunization studies and site-specific mutant proteins for structure-function studies.
8. Continue to attempt to identify the conformational epitope recognized by our crotoxin neutralizing monoclonal antibody using a

variety of chemical, molecular biology, and immunology approaches.

9. Identify mechanisms that snakes use to protect themselves against their own toxins, carry out some characterization of these mechanisms, and determine their medicinal potential.

ACIDIC AND BASIC SUBUNITS OF CROTOXIN

Taxon	Protein	5	10	15	20	25	30
C. d. terrificus	Crotoxin Acidic	. . . . .	. . . . .	. . . . .	. . . . .	S Y G C Y C G A	
C. d. terrificus	Crotoxin Basic	H L L Q F N K M I K P E T R K N A I P F Y A F Y G C Y C G W					

		35	40	45	50	55	60
C. d. terrificus	Acidic	G G Q G W P Q D A S D R C C F E H D C C Y A K L T G C N P T					
C. d. terrificus	Basic	G G R G R P K D A T D R C C F V H D C C Y G K L A K C N T K					

		65	70	75	80	85	90
C. d. terrificus	Acidic	. . . . . pE E D G E I V C G E D D P C G T Q I C G/E C D K					
C. d. terrificus	Basic	W D I Y R Y S L K S G Y I T C G K G T W C E E Q I C E C D R					

		95	100	105	110	115	120
C. d. terrificus	Acidic	A A A I C F R N S M D T . . . . . pE F S P E N C Q G E S Q					
C. d. terrificus	Basic	V A A E C L R R S L S T Y K Y G Y M F Y P D S R C R G P S E					

C. d. terrificus	Acidic	P C
C. d. terrificus	Basic	T C

Fig. 1. Amino acid sequence of the acidic and basic subunits of crotoxin.

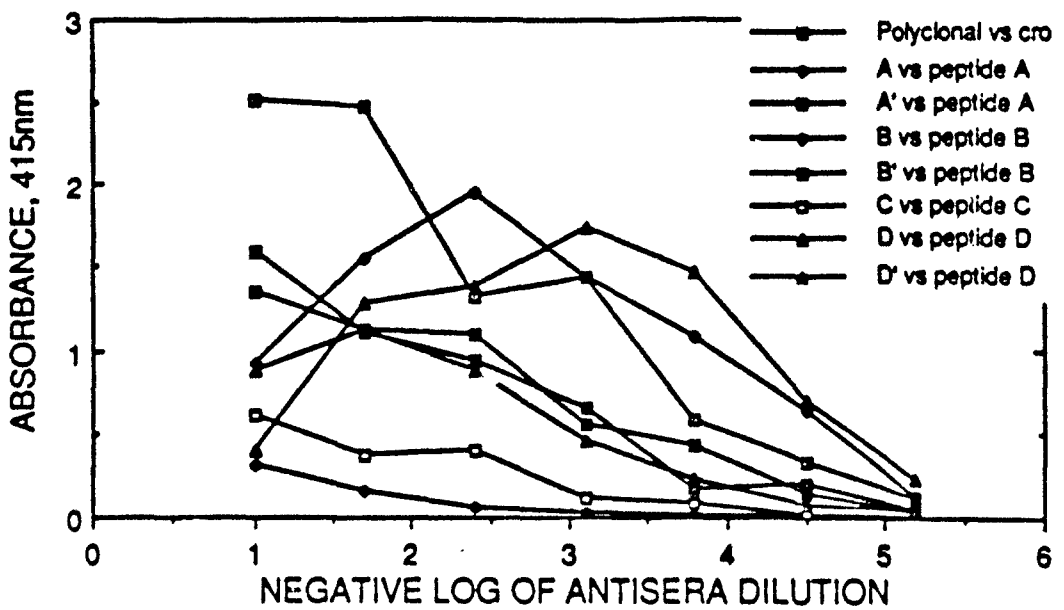

Fig. 2. ELISA titrations of rabbit antisera raised against intact crotoxin and four different peptides, as listed on the right side of the figure, and reacted with their homologous antigens. Peptides A, B, C, and D correspond to sequences 113-122, 102-115, 31-44, and 1-9, respectively, in the basic subunit of crotoxin. These were conjugated to rabbit serum albumin as described in the text and two rabbits were immunized with each conjugate. Plates were coated with homologous antigen and serially diluted rabbit sera assayed for reactivity.

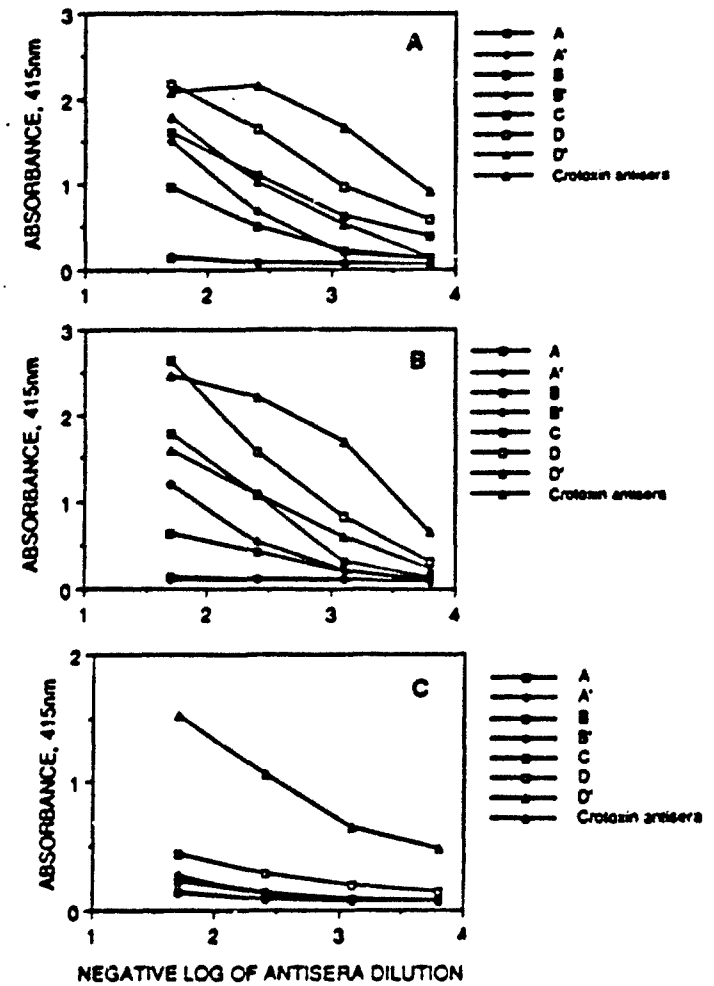

Fig. 3. ELISA cross-reactions with intact crotoxin (A), and its basic (B) and acidic (C) subunits. Plates were coated with either intact crotoxin or one of its subunits and ELISAs were carried out with rabbit antisera raised against the antigens listed to the right of each figure.

pM6 (acidic cDNA) Translated Sequence
Monday, August 24, 1992 2:37 PM

Sequence Range: 1 to 588

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          10         20         30         40         50
     *    *    *    *    *    *    *    *    *    *    *
CAGGG TCTGC TGATT CCCAG GTCTG GATTG AGGAG G ATG AGG GCT CTC TGG
                                             M   R   A   L   W>

          60         70         80         90
     *    *    *    *    *    *    *    *    *    *
ATA GTG GCC GTG TTG CTG GTG GGC GTC GAG GGG AGC CTG GTG GAA TTT
 I   V   A   V   L   L   V   G   V   E   G   S   L   V   E   F>

100        110        120        130        140
  *    *    *    *    *    *    *    *    *    *    *
GAG ACG TTG ATC ATG AAA ATT GCG GGG AGA AGT GGT ATT TCG TAC TAC
 E   T   L   I   M   K   I   A   G   R   S   G   I   S   Y   Y>

150        160        170        180        190
  *    *    *    *    *    *    *    *    *    *    *
AGC TCT TAC GGA TGC TAC TGT GGC GCG GGG GGC CAA GGC TGG CCA CAG
 S   S   Y   G   C   Y   C   G   A   G   G   Q   G   W   P   Q>

   200        210        220        230        240
     *    *    *    *    *    *    *    *    *    *
GAC GCC AGC GAC CGC TGC TGC TTT GAG CAC GAC TGC TGT TAT GCA AAA
 D   A   S   D   R   C   C   F   E   H   D   C   C   Y   A   K>

      250        260        270        280        290
  *    *    *    *    *    *    *    *    *    *    *
CTG ACT GGC TGC GAC CCA ACA ACA GAC GTC TAC ACC TAC AGA CAG GAG
 L   T   G   C   D   P   T   T   D   V   Y   T   Y   R   Q   E>

          300        310        320        330
     *    *    *    *    *    *    *    *    *    *
GAC GGG GAA ATC GTC TGT GGA GGG GAC GAC CCG TGC GGG ACA CAG ATT
 D   G   E   I   V   C   G   G   D   D   P   C   G   T   Q   I>

340        350        360        370        380
  *    *    *    *    *    *    *    *    *    *    *
TGT GAG TGC GAC AAG GCC GCA GCA ATC TGC TTC GCA GAT AGT ATG AAC
 C   E   C   D   K   A   A   A   I   C   F   A   D   S   M   N>

390        400        410        420        430
  *    *    *    *    *    *    *    *    *    *    *
ACA TAC GAC TAC AAA TAT TTG CGG TTC TCG CCC GAA AAT TGC CAG GGG
 T   Y   D   Y   K   Y   L   R   F   S   P   E   N   C   Q   G>

   440        450        460        470        480
     *    *    *    *    *    *    *    *    *    *
GAA TCA CAG CCA TGC TAA GT CTCTG CAGGC GCCGA AAAAC CCCTC AAATT
 E   S   Q   P   C   *>

  490        500        510        520        530        540
    *    *    *    *    *    *    *    *    *    *    *    *
ACACA ATCGT AGTTG TGTTA CTCTA TTATT CTGAA TGCAA TACTG AGTAA TAAAC

       550        560        570        580
  *    *    *    *    *    *    *    *    *    *
AGGTG CCAGC TTGCG GCCGC GAATT CGCGG CCGCG CGGCC GCGAA TTC
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Fig. 4. Sequences of the acidic (A) and basic (B) subunits of Mojave toxin along with the corresponding translated sequences.

pM16 (basic subunit, mtx) Translated Sequence
Monday, August 24, 1992 2:30 PM

Sequence Range: 1 to 542

```
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     *    *    *    *    *    *    *    *    *    *    *
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                                            M   R   A   L   W>

          60         70         80         90
     *    *    *    *    *    *    *    *    *
ATA GTG GCC GTG TTG CTG GTG GGC GTC GAG GGG CAC CTG CTG CAA TTC
 I   V   A   V   L   L   V   G   V   E   G   H   L   L   Q   F>

100        110        120        130        140
  *    *    *    *    *    *    *    *    *    *
AAC AAG ATG ATC AAG TTT GAG ACA AGG AAA AAC GCT ATT CCC TTC TAT
 N   K   M   I   K   F   E   T   R   K   N   A   I   P   F   Y>

150        160        170        180        190
  *    *    *    *    *    *    *    *    *    *
GCC TTT TAC GGC TGC TAC TGT GGC TGG GGG GGC CGA GGC CGG CCA AAG
 A   F   Y   G   C   Y   C   G   W   G   G   R   G   R   P   K>

   200        210        220        230        240
     *    *    *    *    *    *    *    *    *    *
GAC GCC ACT GAC CGC TGC TGC TTT GTG CAT GAC TGC TGT TAC GGA AAA
 D   A   T   D   R   C   C   F   V   H   D   C   C   Y   G   K>

      250        260        270        280        290
  *    *    *    *    *    *    *    *    *    *    *
CTG GCC AAG TGC AAC ACC AAA TGG GAC ATC TAT CCC TAC AGC TTG AAG
 L   A   K   C   N   T   K   W   D   I   Y   P   Y   S   L   K>

        300        310        320        330
     *    *    *    *    *    *    *    *    *
AGT GGG TAT ATC ACC TGC GGA AAG GGC ACC TGG TGC GAG GAA CAG ATT
 S   G   Y   I   T   C   G   K   G   T   W   C   E   E   Q   I>

340        350        360        370        380
  *    *    *    *    *    *    *    *    *    *
TGT GAG TGC GAC AGG GTC GCG GCA GAA TGC CTC AGA AGG AGT CTG AGC
 C   E   C   D   R   V   A   A   E   C   L   R   R   S   L   S>

390        400        410        420        430
  *    *    *    *    *    *    *    *    *    *
ACG TAC AAG TAT GGA TAT ATG TTT TAC CCG GAC TCT CGT TGC AGG GGG
 T   Y   K   Y   G   Y   M   F   Y   P   D   S   R   C   R   G>

   440        450        460        470        480
     *    *    *    *    *    *    *    *    *    *
CCT TCA GAG ACA TGC TAA GT CTCTG CAGGC CGGGA AAACC CCCTC AAATT
 P   S   E   T   C   *>

  490        500        510        520        530        540
    *    *    *    *    *    *    *    *    *    *    *    *
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CG
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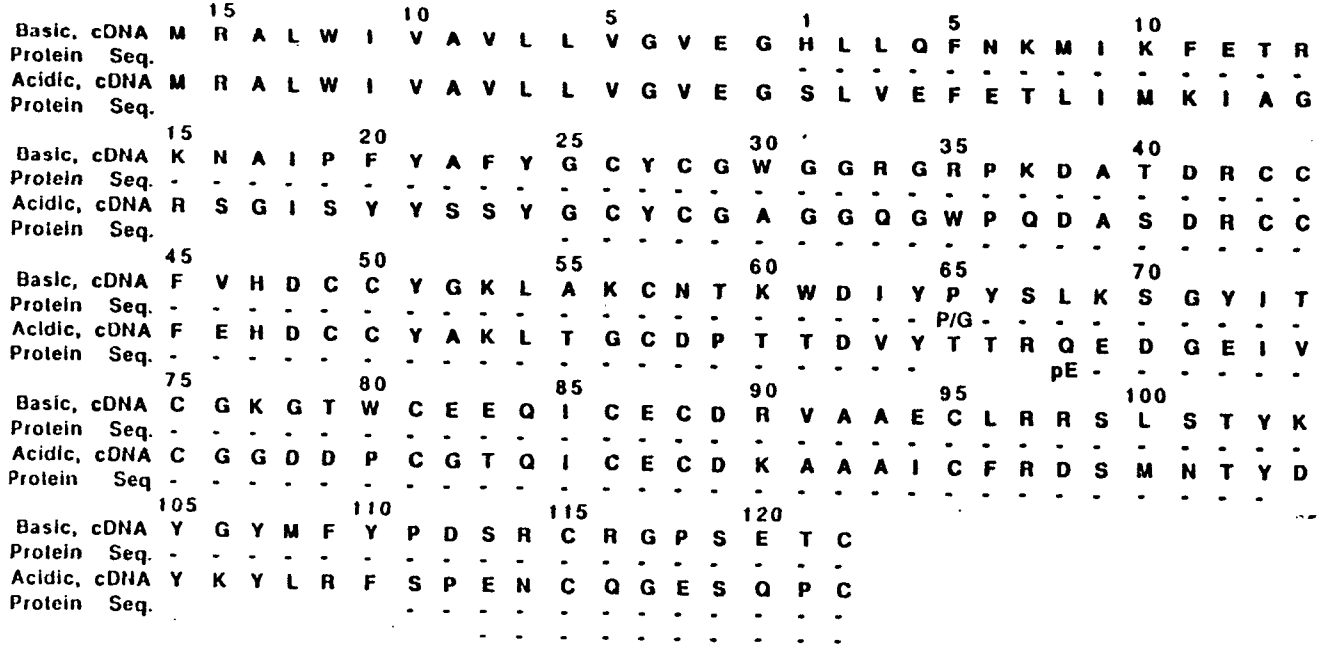

Fig. 5. Sequence comparisons of the cDNAs and proteins for the acidic and basic subunits of Mojave toxin.

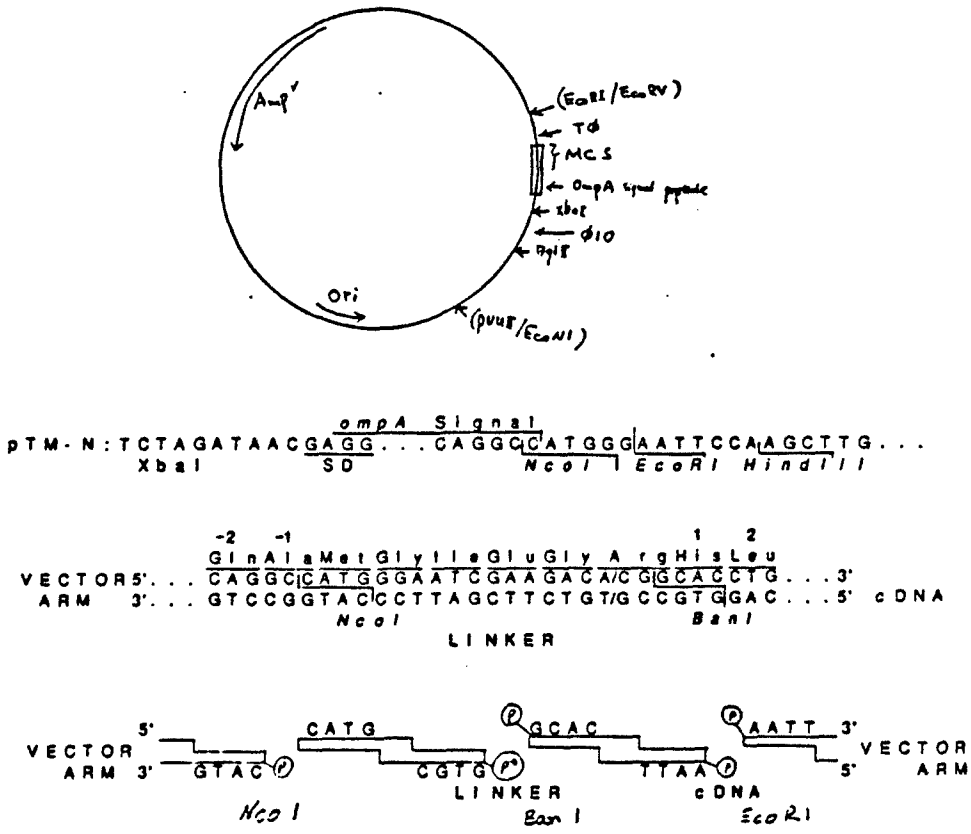

Fig. 6. Construction of pTM-N-bsmt. The BanI/EcoRI cDNA fragment containing the coding sequence for the basic subunit of Mojave toxin, was cloned into pTM-N, immediately downstream from a 18 bp linker. The linker was designed to code for the Factor Xa recognition sequence tetrapeptide, Ile-Glu-Gly-Arg, immediately adjacent to the N-terminal His of the basic subunit protein.

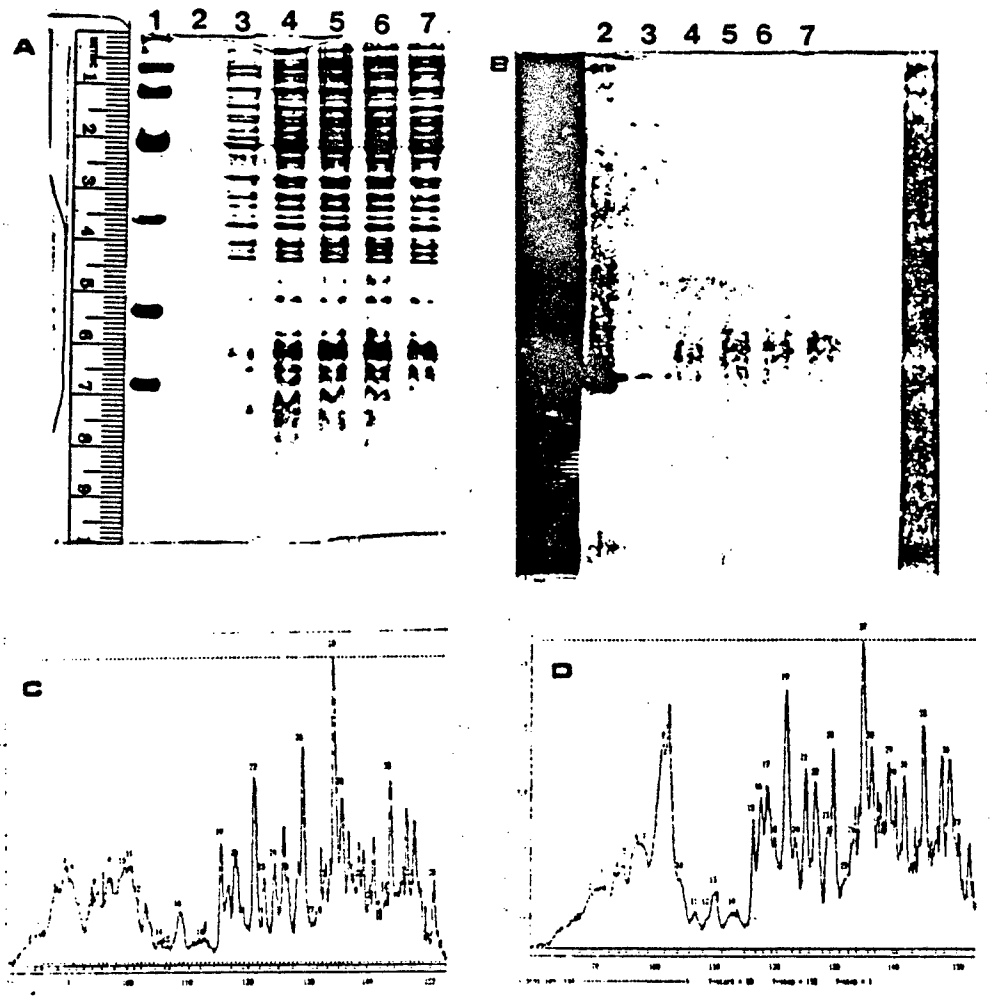

Fig. 7. (A) Coomassie stained SDS-PAGE on 15% gels of E. coli BL21(DE3)(pLysE) samples containing pTM-N-bsmt after induction with 1mM IPTG. Cells were removed at one-hour intervals from zero to four hours, centrifuged, resuspended in protein solubilizing solution and applied to the gel . Lanes: 1, Mr standards; 2, purified, native basic subunit of Mojave toxin; 3, zero; 4, 1 hr; 5, 2 hr; 6, 3 hr; 7, 4 hr. (B) Western blot of a gel identical to (A). Primary antibody used was raised against Mojave toxin. (C and D) Densotometer scans of lanes 3 (zero time) and 7 (4 hr) from panel A. Increase in doublet at position 102 in panel D, represents 3-4% of the total integrated area of the scan.

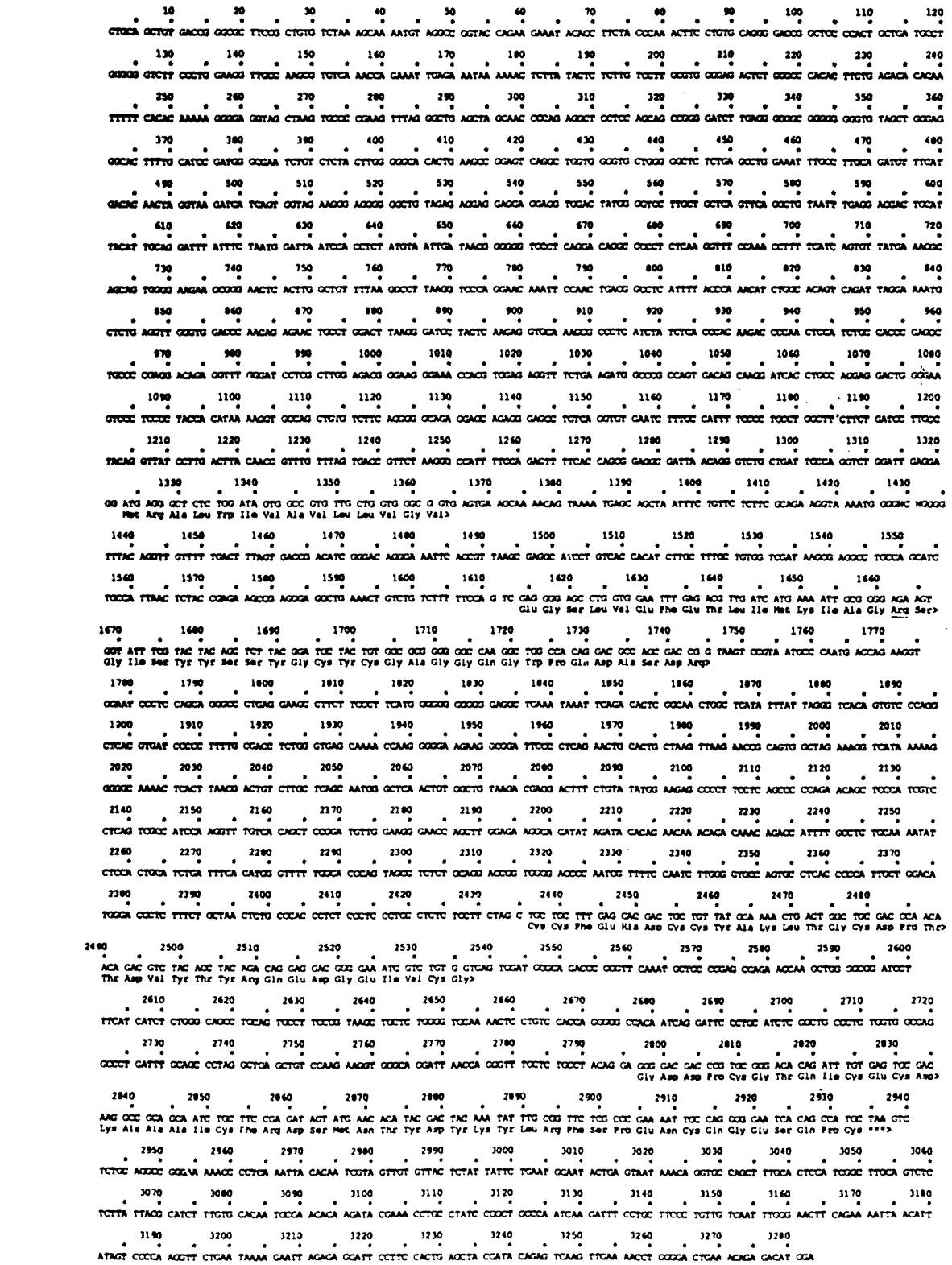

Fig. 8. Acidic subunit gene of Mojave toxin. Translated regions (Exons) have the corresponding amino acids listed under the codons.

Table 1. PEPTIDE ANTIBODY PROTECTION AGAINST LETHALITY

		SURVIVAL TIMES (HOURS)							
		MALE MICE*							No.
ANTISERA	ANTIGEN	< 1	> 1 < 2	> 2 < 3	> 3 < 6	>6 < 24	> 24		Injected
A	Peptide B1		2	2		1			5
A'	Peptide B1		1	1	1	1			4
B	Peptide B2				2	2			4
B'	Peptide B2			2	1	1			4
C	Peptide B3	1		3					4
D	Peptide B4				1	3			4
D'	Peptide B4			1	4				5
T24	Crotoxin						4		4
None				4					4
		FEMALE MICE*							
A+B+C+D		1				4			5
None			1		2	1			4

Table 1. Neutralization experiments consisted of pre-mixing 2µl crude antisera with either 0.1 or 0.13µg intact crotoxin per gram of male or female mouse, respectively, before i.v. injection. Four individual antisera raised against peptides A-D were combined, mixed with crotoxin (0.9µl individual sera or 3.5µl total combined antisera were mixed with 0.13µg intact crotoxin per gram of female mouse), and injected i.v. into mice. LD_{50} value for crotoxin is 0.05µg/g in male and 0.065µg/g in female mice.

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Dr. Laura Degn, post-doctoral associate
Dr. Brian Francis, post-doctoral associate

Dr. Bill Hayes (former graduate student in Zoology, who did about one-half of his experimental work in my laboratory)
Mike Szumski, graduate student in Zoology-Physiology
Noriko Tanaka, current graduate student in Molecular Biology
Nelson daSilva, current graduate in Zoology at BYU in Provo, but has done about 25% of his graduate laboratory work in my laboratory in Laramie.

Don Richie, undergraduate student, now a 4th year medical student
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Keith Spencer, undergraduate student, graduated December, 1991
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